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Tonic and use-dependent block of sodium currents in isolated cardiac myocytes by bisaramil

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1 The effects of bisaramil on sodium currents in rat isolated cardiac myocytes were examined by use of tight-seal, whole-cell patch clamp techniques. Bisaramil produced a concentration-dependent, readily reversible reduction in peak transient sodium current. When the sodium current was evoked at 3 s intervals the estimated ED_{50} for bisaramil was about 11 μM.

2 Bisaramil (16 μM) produced a shift in the inactivation curve to hyperpolarized potentials of about 10 mV, but produced no change in the voltage-dependence of activation.

3 The block of the sodium current by bisaramil showed a profound use-dependence. A concentration of 10 μM produced a considerable block of the current with repeated stimulation. The recovery from block was biphasic, showing fast and slow components which had time constants of about 40 ms and 5 s respectively.

5 Bisaramil produced little tonic block of the sodium current at concentrations of 100 μM; at 300 μM it produced tonic block of around 50%, with extreme use-dependence.

6 Bisaramil appeared not to interact primarily with the inactivated form of the channel, since lengthening the depolarizing pulses did not affect the degree of block produced.

Keywords: Cardiac; transient sodium current; Class I antiarrhythmic; use-dependence; YUTAC

Introduction

Bisaramil (3-methyl, 7-ethyl, 9-α-(4-chlorobenzoyloxy)-3,7-diazabicyclo[3.3.1]-nonane hydrochloride) is an anti-arrhythmic drug developed for the management of ventricular arrhythmias. It has been shown to be effective against both chemical and ischaemic models of arrhythmogenesis (Paroczai *et al.*, 1990; Haruno & Hashimoto, 1993). In isolated cardiac tissue, bisaramil produces an increase in ventricular fibrillation thresholds (Paroczai *et al.*, 1990). In addition it decreases V_{max}, action potential amplitude and overshoot potentials in guinea-pig papillary muscle (Sunami *et al.*, 1991; Paroczai *et al.*, 1992). Electrophysiological studies in multicellular preparations show that bisaramil (3 μM) produces a use-dependent block of the maximum rate of depolarization of the action potential (Sunami *et al.*, 1991). However, there are at present no detailed accounts of the effect of bisaramil on sodium currents in isolated cardiac myocytes.

This study describes the effect of bisaramil on the transient sodium current in rat isolated cardiac myocytes recorded by use of whole-cell clamp techniques. The results of these experiments show that bisaramil can be reasonably described as a drug having strong sodium channel inhibitory effects.

Methods

Isolation of cardiac myocytes

All experiments were performed according to guidelines established by the Animal Care Committee of the Australian National University.

Enzymatic isolation of myocytes was carried out according to the method used previously (Saint *et al.*, 1992). Briefly, male Wistar rats (300-350 g) were killed by cervical dislocation, the hearts removed and washed in ice-cold, calcium-free Tyrode solution for 5 min before Langendorff perfusion at 37°C. After 5 min, enzymatic dissociation was begun in 25 μM calcium Tyrode solution containing protease (0.1 mg ml^{-1}. Sigma Type XIV), collagenase (1 mg ml^{-1}, Worthington CLS II), and foetal calf serum (1 μg ml^{-1}).

After 35 min the ventricles were removed, cut into pieces in fresh 25 μM calcium-Tyrode solution and triturated to dissociate myocytes. Cell suspensions were centrifuged, washed in 200 μM calcium-Tyrode solution, resuspended in 1 mM calcium-Tyrode solution and plated onto glass coverslips. The glass coverslips were transferred to the recording chamber, which had a volume of about 0.5 ml and perfused with recording solution at a flow rate of 0.5 to 1 ml min^{-1}.

Solutions and drugs

All experiments were performed at room temperature (22-25°C) in a bath solution containing (mM): NaCl 70, TES 10, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2, $CoCl_2$ 5, CsCl 5, glucose 10, choline Cl 60, pH adjusted to 7.4 with 1.0 M NaOH. The pipette solution contained (mM): CSF 140, TES 10, $MgCl_2$ 1, K-EGTA 10, $CaCl_2$ 2, ATP-disodium 10 and was adjusted to pH 7.4 with 1.0 M KOH. These solutions are designed to block all ionic currents other than sodium currents. The concentrations of sodium were chosen to reduce the sodium current amplitude, in order to reduce the severity of series resistance problems.

Data recording and analysis

Electrodes were prepared from borosilicate glass using a two-stage puller (Narishige Scientific Instruments, Tokyo, Japan) and resistances were typically between 1-5 MOhm when containing the pipette solution. Myocyte currents were recorded 5-10 min after achieving whole-cell patch clamp configuration (Hamill *et al.*, 1981). Current recording was performed with an Axopatch 200 amplifier (Axon Instruments), with at least 85% compensation of series resistance in all experiments. The amplifier also allowed for initial compensation of both capacitative transients and leak currents. Where necessary, final capacitance and leak compensation

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was performed digitally at the time of analysis by subtraction of the current produced by a 20 mV hyperpolarizing pre-pulse which always preceded the test pulse.

Results

Under whole cell recording conditions, sodium currents were evoked at 3 s intervals by a voltage step to − 40 mV from a potential of − 150 mV. Bisaramil was added to the perfusing solution and sufficient time allowed for steady state block of the current to develop (usually 2 min). Examples of the currents obtained in one cell are shown in Figure 1a. A 50% block of the current was produced by between 8 and 16 μM bisaramil. The concentration-dependence of block is shown in Figure 1b, which shows data from similar experiments in four cells. The estimated ED_{50} from these cells was 11 μM. The block by bisaramil of the sodium current was readily reversible; Figure 1 also shows an experiment in which the magnitude of the current was monitored during a brief application of 30 μM bisaramil. At the stimulation rate used (6 s interval between pulses) onset of block appeared complete in about 1 min, and recovery took about 3 min.

The effects of 16 μM bisaramil on the voltage-dependence of inactivation and activation of the sodium current were also investigated: 16 μM bisaramil produced a shift in the inactivation curve of about 10 mV to hyperpolarized potentials (Figure 2a). This result was confirmed in 4 cells, the mean shift being 12 mV (range 8 to 15). In one cell, 30 μM bisaramil was added; it produced a shift of about 30 mV (data not shown). Bisaramil 16 μM did not produce any shift in the voltage-dependence of activaton of the sodium current, as shown in Figure 2b. This result was also confirmed in 4 cells.

These experiments to estimate the potency of bisaramil were complicated somewhat by the marked use-dependence of the block produced by the drug. Figure 3a shows sodium currents evoked by a train of depolarizations at a frequency of 40 Hz under control conditions and evoked by an identical train of stimuli after 3 min equilibration in 100 μM bisaramil (Figure 3b). No stimuli were given during this equilibration period. The first current of the train was reduced somewhat, by about 20%, indicating some tonic block, and subsequent currents showed a dramatic attenuation with successive stimuli, indicative of a substantial use-dependent block. The concentration-dependence of these effects was investigated by allowing the cell to equilibrate for 3 to 4 min in either 10 μM, 30 μM or 100 μM bisaramil before applying trains of stimuli at 40 Hz. The results are shown in Figure 3c. No frequency-dependent attenuation of the current was apparent in the absence of bisaramil under these conditions. Increasing concentrations of bisaramil produced increasing degrees of attenuation of the peak current amplitude with successive stimuli, although very little tonic block was produced, as

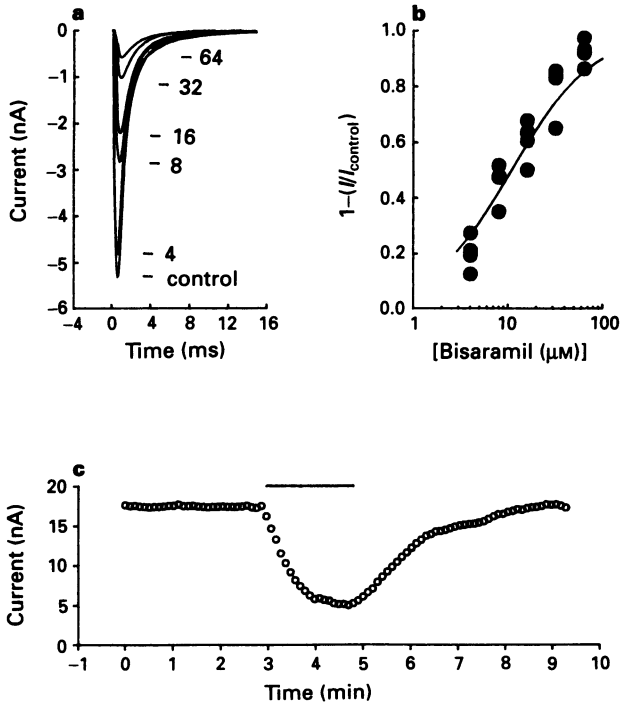

Figure 1 Concentration-dependent block of the transient sodium current by bisaramil. Currents were evoked at 3 s intervals by a voltage step to − 40 mV from a pre-pulse potential of − 150 mV. (a) Shows currents obtained under control conditions and with various concentrations of bisaramil (shown by markers, in μM); (b) shows the data from 4 cells plotted as normalized block of peak current amplitude against concentration of bisaramil; (c) shows the time course of onset and recovery from block induced by bisaramil. Sodium currents were evoked at 6 s intervals. At the time indicated by the bar, 30 μM bisaramil was added to the solution perfusing the cells. The maximum sodium current amplitude is shown plotted against time.

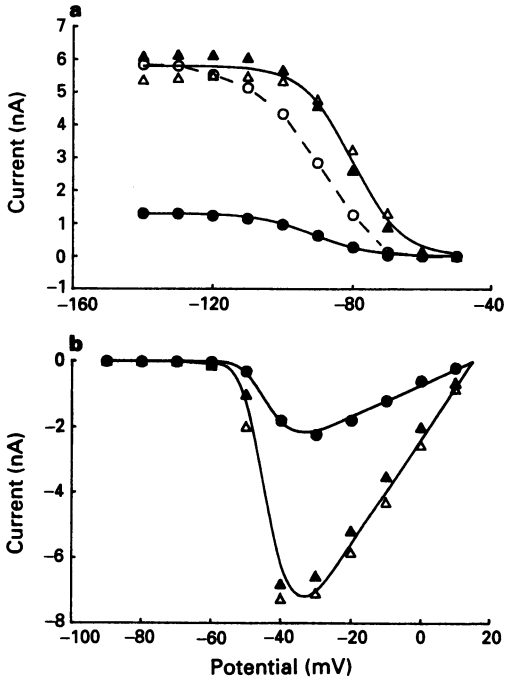

Figure 2 Effect of bisaramil on the voltage-dependence of the sodium current. (a) Shows the voltage-dependence of inactivation of the sodium current under control and recontrol conditions (▲ and △) and in the presence of 16 μM bisaramil (●). Sodium currents were evoked by voltage pulses to − 30 mV from various pre-pulse potentials. The data are plotted as the peak current amplitude against pre-pulse potential. The lines show the fits of the equation

$$I = I_{max}/1 + \exp((v\text{-}v')/k)$$

with values of I_{max} = 5.8 nA, v′ = − 80 mV and k = 8 for control/recontrol and I_{max} = 1.3 nA, v′ = − 91 mV and k = 7 for bisaramil. (○) Bisaramil data scaled to the same maximum current as the control/recontrol. (b) Shows the current-voltage relation for the sodium current in control/recontrol and with 16 μM bisaramil. Sodium currents were evoked by depolarizing pulses to various potentials from a fixed pre-pulse potential of − 140 mV. The data are plotted as peak current amplitude against pulse potential. The conventions for symbols are as in (a). The lines are the best fits of the equation

$$I = G_{max}/1 + \exp((v\text{-}v')/k)x(E_{rev}\text{-}v)$$

with values of E_{rev} = 15 mV, k = 3.5 and v′ = − 43 mV for both sets of data. G_{max} was 16 nS for control/recontrol and 4.8 nS for bisaramil.

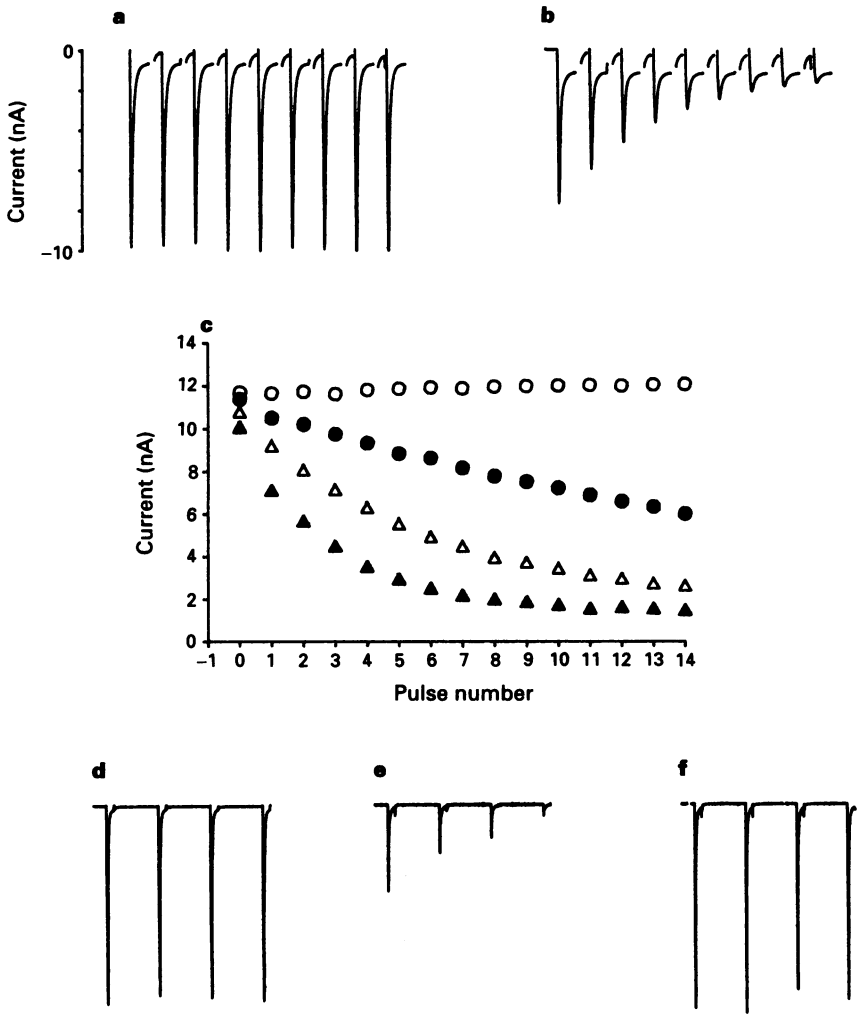

Figure 3 Use-dependent block and tonic block by bisaramil at different concentrations. (a) Shows sodium currents evoked by a train of pulses to − 40 mV from a potential of − 120 mV, delivered at 40 Hz. After equilibration for 3 min in solution containing 100 μM bisaramil, an identical train of stimuli evoked the currents shown in (b). Pronounced attenuation of the current was produced with successive stimuli. (c) Shows the effect of different concentrations of bisaramil upon the currents evoked by trains of stimuli at 40 Hz. The peak sodium current for each stimulus in the train is plotted against pulse number. The plots show control data (○), and data in the presence of 10 μM (●), 30 μM (△) and 100 μM (▲) bisaramil. The cell was equilibrated in each concentration of bisaramil for 3 min before the train of stimuli was delivered. (d) Shows the sodium currents evoked in a different cell by a train of four stimuli at 20 Hz in control solution. In (e) are shown currents evoked by an identical train given after the cell had been equilibrated for 8 min in solution containing 300 μM bisaramil. The currents evoked after 9 min in recontrol solution are shown in (f).

long as sufficient time was allowed for recovery between trains. In a few experiments 300 μM bisaramil was applied to the cells. A typical example of the results is shown in Figure 3d. After 8 min equilibration in 300 μM bisaramil in the absence of any stimulation, the peak current amplitude of the first current evoked was reduced by about 50% (Figure 3e). The use-dependence of the block by this concentration was very pronounced; after only four pulses at 20 Hz the current was essentially abolished. In one experiment 500 μM bisaramil was applied to the cell. Even after 10 min in this solution, a sodium current could be evoked. Frequency-dependent block was extreme at this concentration.

In these experiments it became apparent that recovery from block by bisaramil took some considerable time. This is illustrated by the result shown in Figure 4a, in which two trains of stimuli at 20 Hz were delivered with an interval between them of 4 s. The bathing solution contained 100 μM bisaramil. A rapid diminution of the current was seen during the first train, but at the beginning of the second train recovery from this block was minimal, despite the 4 s gap between trains. This result suggested that the frequency-dependence of block by bisaramil had a slow component. This slow phase of frequency-dependent block is illustrated in Figure 4b, which shows the attenuation of the current by trains of stimuli of a wide range of frequencies in the presence of 100 μM bisaramil. As expected, higher frequencies of stimulation produced a more rapid block of the sodium current (Figure 4c). However, it was apparent that even stimulation rates as low as pulses delivered at 10 s intervals produced a substantial block of the current.

In order to study this frequency-dependence further, experiments were performed in the presence of 30 μM bisaramil, which allowed analysis at higher stimulation rates.

Figure 5 shows the block of the current produced by successive stimuli for trains of stimuli with frequencies of between 0.5 and 80 Hz. At the slower stimulation rates, between 0.5 and 12 Hz, there was little difference discernible in the attenuation of the current with successive stimuli. This suggests that, at these stimulation rates, the interval between pulses is short compared to the rate of recovery from block. However, at higher rates of stimulation, i.e., 70 or 80 Hz, the attenuation of the current with successive pulses becomes

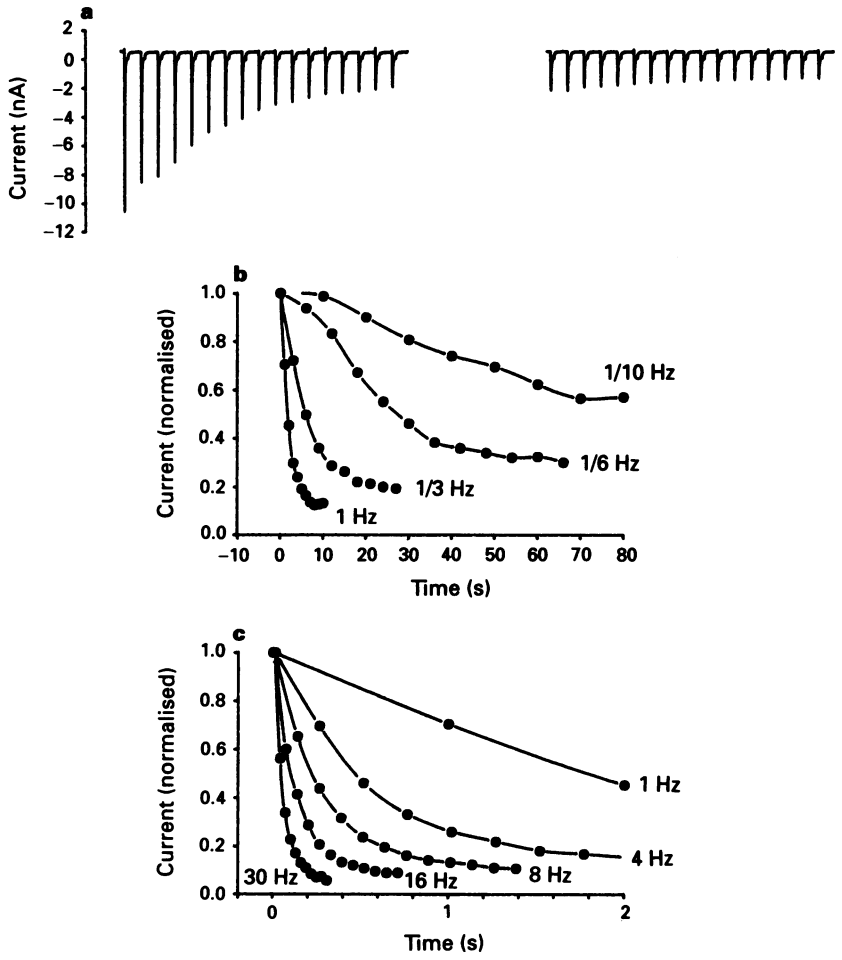

Figure 4 Use-dependent block by bisaramil over a wide range of frequencies. In (a) the sodium currents evoked by a train of depolarizing pulses from − 120 mV to − 40 mV delivered at 20 Hz in solution containing 100 μM bisaramil are shown. The train was repeated after a 4 s delay, yielding the currents shown after the break in the baseline. (b and c). Trains of pulses to − 40 from − 120 mV were delivered at various frequencies ranging between 1/10 Hz and 30 Hz. The data are plotted as the peak current amplitude (normalized to the first current size) against the time at which it was evoked. Trains of stimuli were delivered at 1/10 Hz, 1/6 Hz, 1/3 Hz, 1 Hz (shown in b) and 4 Hz, 8 Hz, 16 Hz and 30 Hz (shown in c on an expanded time scale).

more pronounced. This result could arise if there was a second kinetic component to the recovery from block with a shorter time constant.

The rate of recovery from block was therefore investigated more thoroughly with a double-pulse type methodology. Sodium currents were evoked by pairs of identical depolarizing pulses, the interval between the pulses being varied. The size of the current evoked by the second pulse was then plotted against the interval between the pulses, to give a measure of the rate of recovery from block. Such an experiment is illustrated in Figure 6. The rate of recovery from the block in these experiments could never be adequately fitted with a single exponential. However, the recovery could be well described by the equation

$$1-[\beta x(\alpha/e^{t/\tau_1}) + ((1-\alpha)/e^{t/\tau_2})]$$

which describes recovery from an incomplete block as the sum of two exponentials. For the data shown, a reasonable fit was obtained with $\beta = 0.65$, $\alpha = 0.6$, $\tau_1 = 40$ ms and $\tau_2 = 5$ s. A similar analysis in 3 more cells gave values for the time constants of recovery of between 20 and 50 ms for the fast recovery, and 3 and 8 s for the slow recovery. It should be noted, however, that this method of estimating recovery time constants is not accurate, since a unique solution to equation 1 cannot generally be found. Nevertheless, it was clear from the data that two exponential components having widely different time constants were always necessary to describe the recovery from block.

The use-dependence of block could arise because of an interaction of bisaramil with either the open channel, or the inactivated channel. This question was addressed by giving pairs of voltage pulses to evoke sodium currents and varying the duration of the first pulse, as shown in Figure 7. Prolonging the depolarization does not change the length of time for which the channels are open, but a prolonged depolarization holds the channels in the inactivated state for a longer period of time. If the drug interacts with the inactivated form of the channel, one would therefore expect an increased degree of block with prolonged depolarizations. As shown in Figure 7, this did not occur; prolonging the depolarization from 5 ms to almost 50 ms did not substantially affect the degree of block of the subsequent current.

Discussion

The data show that bisaramil produces a concentration-dependent, readily-reversible block of the transient sodium current in cardiac myocytes and shifts the inactivation curve for the sodium current to more hyperpolarized potentials, although no shift in the activation curve was apparent. This spectrum of actions is very similar to that seen with class I anti-arrhythmic agents, eg. lignocaine (Bean *et al.*, 1983) and quinidine (Snyders & Hondeghem, 1990).

These results corroborate earlier suggestions, from studies performed in intact animals and isolated cardiac tissues, that

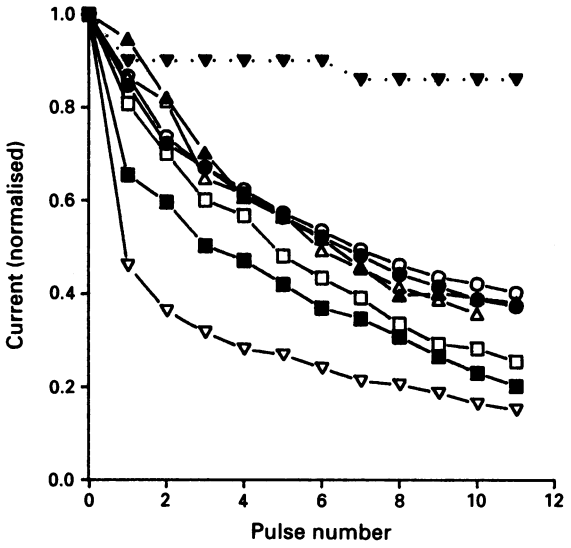

Figure 5 Use-dependence of block at higher frequencies. Trains of pulses to − 40 mV from − 120 mV were given in a solution containing 30 μM bisaramil. The magnitude of the current evoked by each pulse is plotted against the pulse number. The currents are plotted as a fraction of the first current in each train. The trains of pulses at frequencies of 0.5 Hz, 1 Hz, 5 Hz and 12 Hz (○, ●, △, ▲) produced use-dependent block which was indistinguishable. At a frequency of 21 Hz, however, a noticeably more pronounced block was produced (□), and this block was accentuated at frequencies of 70 Hz, (■) and 90 Hz (▽). In the same cell in the absence of bisaramil there was no attenuation of current amplitude at frequencies up to 90 Hz (▼).

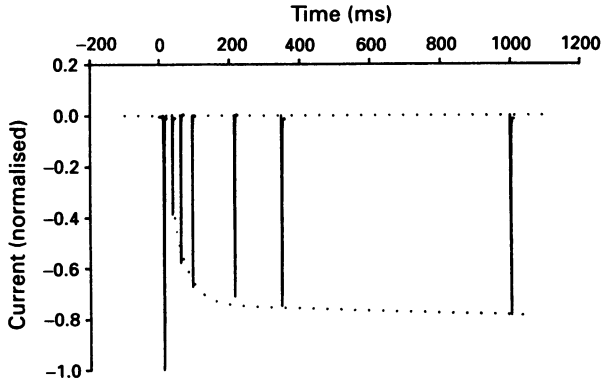

Figure 6 Biphasic recovery from block. Pairs of depolarizing pulses were delivered in a solution containing 100 μM bisaramil, with a variable delay between pulses. The currents evoked are shown plotted at the time that they were evoked, normalized to the size of the first current. The dotted line shows the fit of the equation

$$1-[\beta.(\alpha/e^{t/\tau_1}) + ((1-\alpha)/e^{t/\tau_2})]$$

to the peak current amplitude with $\beta = 0.65$, $\alpha = 0.6$, $\tau_1 = 40$ ms and $\tau_2 = 5$ s.

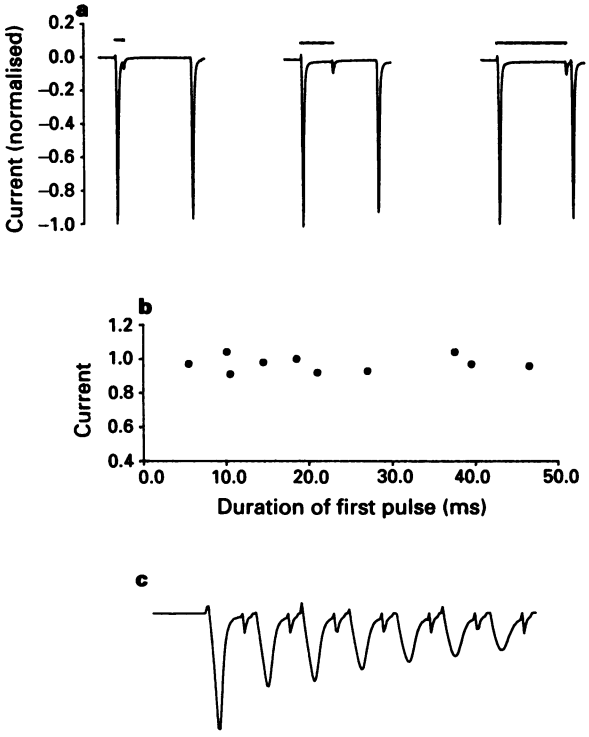

Figure 7 The effect of changing the duration of depolarization. Pairs of depolarizing pulses were given with a delay of 50 ms between the pulses. The duration of the first pulse was varied over the range 5 ms to 46 ms. The currents evoked are shown (a), with the first current magnitude normalized. The bar above the current trace shows the duration of the first depolarization. (b) The magnitude of the second pulse, as a fraction of the first, is plotted against the duration of the first pulse. There was no correlation of the second current size with duration of the first pulse. (c) In the same experiment, a train of stimuli at 40 Hz produced an obvious frequency-dependent block.

bisaramil probably produces its anti-arrhythmic actions via blockade of cardiac sodium channels (Paroczai *et al.*, 1990; 1992; Haruno & Hashimoto, 1993).

Use-dependence of the block by bisaramil has been reported by Sunami *et al.* (1991), in experiments in which action potentials were recorded from multicellular preparations. The data presented here in single myocytes confirm that bisaramil shows a marked frequency-dependent block, which can be dissected into a fast and a slow component. The two components of frequency-dependence appear to arise from a biphasic recovery from block, which occurs with a mixture of a fast recovery, having a time constant of the order of a few tens of milliseconds, and a slow recovery with a time constant of the order of seconds. In practice, the presence of the very slow component of recovery means that care must be exercised in the interpretation of results such as dose-response curves, since the presence of the slow component means that a substantial use-dependent block is present, even when the sodium current is evoked at 3 s intervals. Because of this very slow component of recovery from block, it was very difficult to obtain an estimate of the tonic block produced by bisaramil, since one would have to evoke the sodium current at such a slow rate that time-dependent changes, which are inevitable in patch clamp experiments, obscure the results. Nevertheless, the data suggest that the degree of tonic block produced by bisaramil is small, at least at concentrations encountered in animal experiments, since exposing the cell to 100 μM bisaramil for several minutes attenuated the magnitude of the first pulse in the train by only about 10%. Exposure of the cells to bisaramil 300 μM for several minutes attenuated the first current evoked by 50%. At this concentration of bisaramil the use-dependence of block was such that the current was completely blocked after only three depolarizing pulses were given.

On the basis of the data presented here we conclude that bisaramil produces its antiarrhythmic actions primarily by blocking the transient sodium current and hence can be classified as a class I agent. Use-dependence of block is a useful property for an anti-arrhythmic agent, since rapid firing of action potentials will be suppressed, but the normal firing of action potentials will not be greatly affected. The biphasic frequency-dependence of block by bisaramil may be advantageous in this respect.

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